

Purification and characterization of mouse mast cell proteinase-2 and the differential expression and release of mouse mast cell proteinase-1 and -2 *in vivo*

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Summary

Background Gastrointestinal nematode infection is associated with mucosal mast cell (MMC) hyperplasia. In the mouse, this is accompanied by the release of substantial quantities of the chymase mouse mast cell proteinase-1 (mMCP-1) into the gut lumen and peripheral bloodstream. Expression of mMCP-1 is largely restricted to intraepithelial MMC and is thought to play a role in the regulation of epithelial permeability. MMCs also express mouse mast cell proteinase-2 (mMCP-2), but less is known about the expression or biological function of this proteinase.

Objectives (1) To purify and characterize mMCP-2. (2) To compare the expression and release of mMCP-2 and mMCP-1 *in vivo* using specific antibodies.

Methods Bone marrow-derived mast cells (mBMMCs) were generated from mMCP-1^{-/-} BALB/c mice. mMCP-2 was purified, characterized and used to generate rat and sheep polyclonal antibodies. The expression and systemic release of mMCP-1 and -2 were compared *in vivo* by immunohistochemistry and ELISA.

Results mMCP-2 was successfully purified from mMCP-1^{-/-} mBMMC and its identity confirmed by N-terminal amino acid sequencing. mMCP-2 bound [3H]-labelled DFP, indicating the presence of an active serine proteinase catalytic site, but showed little evidence of chymotryptic activity. MMC expressed comparable levels of mMCP-1 and -2 in the jejunum but not in the gastric mucosa, where mMCP-2 was more abundant. Expression of both proteinases increased substantially during primary *Nippostrongylus brasiliensis* infection and this was accompanied by a substantial increase in peripheral blood levels of mMCP-1 (70 µg/mL on day 12). By contrast, mMCP-2 was not detected in the serum of uninfected mice and only increased to approximately 25 ng/mL on day 12.

Conclusion As in the case of mMCP-1, mMCP-2 expression is restricted to MMC. However, mMCP-2 lacks chymase activity, is expressed at higher levels in gastric MMC and appears to be differentially released into the peripheral bloodstream.

Keywords bone marrow, chymase, gastric mucosa, intestinal mucosa, *Nippostrongylus brasiliensis*, TGF-β1

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Introduction

Infection with gastrointestinal helminth parasites typically elicits a Th2 cytokine response and recruitment of abundant mast cells to the gut mucosa [1]. Two possible mechanisms of recruitment have been described in the mouse. The first involves the migration of mast cell precursors into the epithelium [2, 3], where they differentiate and express the mucosal mast cell (MMC)-specific chymase mMCP-1 [4–6]. The second proposed mechanism involves the migration of

submucosal mast cells to the villus epithelium, changing the expression of mouse mast cell proteinases (mMCPs) as they progress. In particular, expression of the tryptases mMCP-6 and -7, and the putative chymase mMCP-5 cease on migration into the mucosa, and mMCP-1 and -2 are expressed in their place [7]. During *Trichinella spiralis* infection, mMCP-1 is secreted into the gut lumen in high concentrations [8], and can be detected systemically in the bloodstream in a complex with serpins [9]. It is likely that the proteolytic activity of mMCP-1 and/or -2 plays a role in the permeability increase of the jejunal epithelium, which may contribute to parasite rejection [1]. In support of this hypothesis, gene deletion of mMCP-1 [10] results in delayed expulsion of *T. spiralis* in knockout mice [11].

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Of the five designated mouse mast cell chymases (mMCP-1, -2, -4, -5 and -9) [12, 13], only mMCP-1 and -4 have been shown to be functional chymases [14, 15]. mMCP-4 is a β -chymase that can be purified from peritoneal mast cells [15] and is analogous to rat mast cell proteinase-1 [16]. It is highly basic with activity towards chromogenic substrates and converts angiotensin-I to -II by rapid cleavage at Phe(8) [17], a property shared with human α -chymase [18]. Multiple glycoforms of mMCP-1 have been isolated, and all found to be active in terms of hydrolysing synthetic substrates [15], although no physiologically relevant substrates have so far been suggested. Although mMCP-2 was originally identified and cloned by Serafin et al. [19] in 1990 and shown to bind the serine proteinase inhibitor diisopropyl fluorophosphate (DFP), it has never been purified, and its substrate specificity has not been defined. Intriguingly, the murine α -chymase, mMCP-5, was recently expressed as a recombinant protein and was shown to have elastase-like rather than chymase-like properties [20]. This is a clear indication that the mast cell 'chymase' family is more functionally heterogeneous than was originally thought.

We have recently shown that mouse bone marrow-derived mast cells (mBMMCs) grown in the presence of TGF- β_1 are highly analogous to MMC and express abundant mMCP-2 mRNA transcripts [21, 22]. In the current study, we have used the TGF- β_1 culture system [21, 22] to generate MMC-homologues from mMCP-1^{-/-} mice [10, 11] that express mMCP-2 in the total absence of mMCP-1. After isolating and confirming the identity of mMCP-2, we have generated highly specific polyclonal antibodies with which to study the expression and release of this proteinase in more detail. Our results confirm that the expression of mMCP-2 is restricted to MMC, but show that it differs significantly from mMCP-1 in its distribution, chymotryptic activity and appears to be released into the peripheral bloodstream in much lower amounts. The possibility that mMCP-2 expression is, like mMCP-1 [21], regulated by local TGF- β_1 expression within the mucosa is discussed.

Material and methods

Generation of MMC-homologues

Bone marrow cells (BMCs), prepared from 10–12-week old male mMCP-1^{-/-} [10, 11] or wild-type BALB/c mice as described previously [21, 22], were washed twice in DMEM (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Serotec, Kidlington, UK), 100 U/mL penicillin, 100 μ g/mL streptomycin, 2.5 μ g/mL fungizone, 2 mM L-glutamine and 1 mM sodium pyruvate (DMEM/FCS). Nucleated cells were counted in an Improved Neubauer counting chamber (Fisher Scientific, Loughborough, UK) after dilution in white blood cell counting fluid (Sigma, Poole, UK). Viability was assessed using 0.2% nigrosin (Sigma) exclusion. BMC cultures were set up at 5×10^5 viable cells/mL in TGF- β_1 /IL-3/IL-9/SCF (DMEM/FCS supplemented with 1 ng/mL recombinant human TGF- β_1 (TGF- β_1 : Sigma), 1 ng/mL recombinant mouse IL-3 (IL-3: R&D Systems Abingdon, UK), 5 ng/mL recombinant mouse IL-9 (IL-9: R&D Systems), and 50 ng/mL recombinant mouse SCF (PeproTech EC Ltd, London, UK) [21, 22].

Cultures were maintained in a humidified 5% CO₂ incubator and fed with fresh TGF- β_1 /IL-3/IL-9/SCF every 2–3 days for 14 days *ex vivo*.

Purification of mMCP-1 and -2 from mBMMC

All procedures were carried out at 0–4 °C, apart from chromatography, which was performed at room temperature.

For the purification of mMCP-1, approximately 1.5×10^8 BALB/c MMC-homologues were freeze-thawed five times and vortexed in 2 mL of 20 mM Tris-HCl (pH 7.5). After centrifugation at 20 000 *g* for 30 min, mMCP-1 was purified from the soluble extract using the conditions described by Newlands et al. [15], except that mMCP-1 was not sub-fractionated into individual glycoforms. Some mMCP-1 preparations were further purified by heparin affinity chromatography (Hi-Trap Heparin, 1 mL, Amersham) in 50 mM MES, pH 6.0, eluting with a 0–0.3 M NaCl gradient.

For the purification of mMCP-2, frozen pellets from approximately 1.5×10^8 mMCP-1^{-/-} MMC-homologues were homogenized with 5 mL of 50 mM MES (pH 6.1), 0.5 M NaCl and 0.1% Brij 35. After centrifugation, filtration and dilution of the supernatant with 50 mM MES (pH 6.1) and 0.1% Brij 35, it was purified by two ion-exchange chromatography steps (Mono S, Amersham Biosciences, Little Chalfont, UK) firstly in 50 mM MES (pH 6.1), 0.1% Brij 35, then in 20 mM Tris (pH 7.5), 0.1% Brij 35. Fractions containing abundant protein of the expected MW for mMCP-2 [19] were combined, concentrated (Centricon YM-10, Millipore, Watford, UK), then applied to a gel filtration column (Superdex 75, Amersham Biosciences) in PBS, 0.1% Brij 35. The fractions comprising the major peak of purified mMCP-2 were stored at –70 °C. The identity of the product was verified by N-terminal amino acid sequencing of material run on SDS-PAGE and transblotted to PVDF membrane (P. Barker, Babraham Institute, Cambridge, UK).

Analysis of mMCP-2 substrate specificity

Purified mMCP-2 was incubated with [1,3-³H]DFP to test for the presence of an active serine proteinase, and equine trypsin [23] was used as a positive control. Samples were separated by SDS-PAGE, and gels exposed to film as previously described [24].

The activity of mMCP-2 was screened by incubation with chromogenic substrates (Sigma) for elastase: succinyl-Ala-Ala-Ala-*p*-nitroanilide, methoxysuccinyl-Ala-Ala-Pro-Met-*p*-nitroanilide and methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; for chymotrypsin: succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester; for trypsin: carbobenzyloxy-Lys-thiobenzyl ester, toluenesulphonyl-Gly-Pro-Lys-*p*-nitroanilide and benzoyl-Val-Gly-Arg-*p*-nitroanilide; and for aspartase: succinyl-Ala-Ala-Pro-Asp-*p*-nitroanilide.

In each case, 10 μ L of 75 μ g/mL mMCP-1 or -2 was added to 190 μ L reaction mixture containing the substrate at 0.5 mM final concentration, plus 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) at 0.5 mM when thioester substrates were used. Incubation buffers were 50 mM Tris pH 6.8, 50 mM HEPES pH 7.5 and 50 mM Tris pH 8.8, each containing 0.15 M NaCl.

Results were corrected for background hydrolysis in the absence of enzyme.

A further comparison of the chymotryptic activity of mMCP-2 against other known chymotryptic proteinases was carried out under buffer conditions optimal for their activity: 0.5 mM succinyl-Ala-Ala-Pro-Phe thiobenzyl ester, 0.1 mM DTNB, 1.5 M NaCl, 0.3 M Tris-HCl, pH 8.0. Human chymase was purified from skin tissue as previously described [25]; human neutrophil cathepsin G and human seminal fluid prostate-specific antigen (enzymatically active) were purchased from Calbiochem (Nottingham, UK).

The comparative hydrolysis of resorufin-labelled casein (Universal Protease Substrate, Roche, Welwyn Garden City, UK) by mMCP-1 or -2 (both 38 µg/mL final concentration) and PBS (control) was carried out using the manufacturer's protocol (except that incubation was for 2 h at 37 °C in PBS). Released resorufin was detected by UV absorbance (574 nm at pH 8.8) following TCA precipitation of unreacted substrate.

Preparation of anti-mMCP-2 polyclonal antibodies

Wistar rats were inoculated with 10 µg mMCP-2 in Freund's complete adjuvant, boosted twice with 10 µg mMCP-2 in Freund's incomplete adjuvant, and bled 10 days after the last boost. Antiserum was also raised in a sheep using a similar protocol, with 50 µg mMCP-2 for the initial inoculation and two boosts with 25 µg mMCP-2. Rat immunoglobulins were purified from the rat anti-mMCP-2 antiserum using a Protein G column (Hi-Trap Protein G, Amersham Biosciences), then cross-absorbed against mMCP-1 conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences). Sheep anti-mMCP-2 antiserum was first cross-absorbed against mMCP-1 (as above), then specific anti-mMCP-2 Ig were purified using an affinity column (Hi-Trap NHS-activated, 1 mL, Amersham Biosciences) to which mMCP-2 (0.45 mg) had been coupled according to the manufacturer's instructions. Antibody elution was achieved using 0.1 M citric acid, followed by addition of 1 M Tris-base to pH 7.5.

Immunohistochemical staining of mouse tissues with anti-mMCP-1 and -2 antibodies

Tissues were fixed in 4% paraformaldehyde in PBS for 6 h and processed into paraffin blocks. Four-micrometre sections were mounted on Snow Coat X-tra™ charged slides (Surgipath Europe, Peterborough, UK), dewaxed in xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked by a 20-min incubation in methanol containing 3% H₂O₂. Anti-mMCP-1 staining was performed as described previously using the rat monoclonal anti-mMCP-1 antibody Mab RF6.1 [6]. For anti-mMCP-2 staining, sections were incubated with 1 µg/mL of cross-absorbed rat anti-mMCP-2 Ig, 1 µg/mL of cross-absorbed affinity-purified sheep anti-mMCP-2 Ig or 1 µg/mL of control rat or sheep Ig. Dilutions were made in PBS (pH 7.4) containing 0.5 M NaCl and 0.5% Tween-80. Following incubation for 1 h at 21 °C and washing with PBS, sections were incubated for 30 min with biotinylated rabbit anti-rat IgG (1:100: dilution: Vector Laboratories, Peterborough, UK) or biotinylated donkey anti-sheep IgG (1:1000: Jackson

ImmunoResearch Laboratories, West Grove, PA, USA) as appropriate. Sections were then treated with avidin-horse-radish peroxidase conjugate (ABC kit, Vector Laboratories), followed by diaminobenzidine (DAB kit, Vector Laboratories). Finally, sections were lightly counterstained with haematoxylin, dehydrated through graded alcohol, cleared in xylene and mounted in DPX.

ELISA for mMCP-1 and -2

mMCP-1 ELISA assays were performed as described previously [11]. For the mMCP-2 ELISA, Dynatech M129B 96-well plates were coated overnight at 4 °C with 1 µg/mL of affinity-purified sheep anti-mMCP-2 Ig in carbonate buffer (pH 9.6). Wells were washed ($\times 6$) with 0.15 M NaCl, 0.05% Tween-20. Standards (0.1–5.0 ng/mL of purified mMCP-2) and samples were applied (50 µL/well), diluted as appropriate in PBS containing 4% BSA and 0.05% Tween-20. After incubation at 37 °C for 1 h, plates were washed as above and incubated for 1 h at 37 °C with 5 µL/well of rat anti-mMCP-2 Ig (0.5 µg/mL in PBS containing 4% bovine serum albumin (BSA) and 0.05% Tween-20). Plates were washed and incubated with 50 µL/well of biotinylated rabbit anti-rat IgG (Vector Laboratories; 1:500) for 30 min at 37 °C, then washed and incubated with 50 µL/well streptavidin-horse-radish peroxidase conjugate (Amersham Biosciences, 1:2000) for 30 min at 37 °C. Finally, plates were washed and 50 µL/well of TMB substrate (Insight Biotechnology Ltd, Wembley, UK) was added. After sufficient colour development had taken place, a further 50 µL/well of 0.18 M sulphuric acid was added to stop the reaction. Plates were read at 450 nm on a Model 550 Microplate Reader (Bio-Rad Laboratories, Hemel Hempstead, UK). The standard curve was generated using a cubic curve fit and sample concentrations were calculated using Microplate Manager 4.0 software (Bio-Rad Laboratories).

Nippostrongylus brasiliensis infection of BALB/c mice

Age-matched female BALB/c mice ($n = 10$) were infected subcutaneously with 500 *N. brasiliensis* L₃ larvae as described previously [11]. Fecal egg counts were performed daily. Five uninfected littermates were killed on day 0, and infected mice were killed on days 7 ($n = 5$) and 12 ($n = 5$). Samples of stomach and jejunum were frozen on dry ice for ELISA and fixed in 4% paraformaldehyde for immunohistochemistry. In jejunal sections, positively stained mast cells were enumerated per 20 villus-crypt units (VCU) both in the mucosa and in the submucosa directly below [11]. In sections of the stomach, positively stained mast cells were counted in 10 adjacent fields in the glandular fundus and enumerated per mm² [6]. All data were compared using the non-parametric Mann-Whitney test (Instat 3.0: GraphPad Software Inc., San Diego, CA, USA).

Results

Purification of mMCP-2 from mMCP-1^{-/-} MMC-homologues

In order to isolate mMCP-2, in the absence of contaminating mMCP-1, we purified mMCP-2 from MMC-homologues

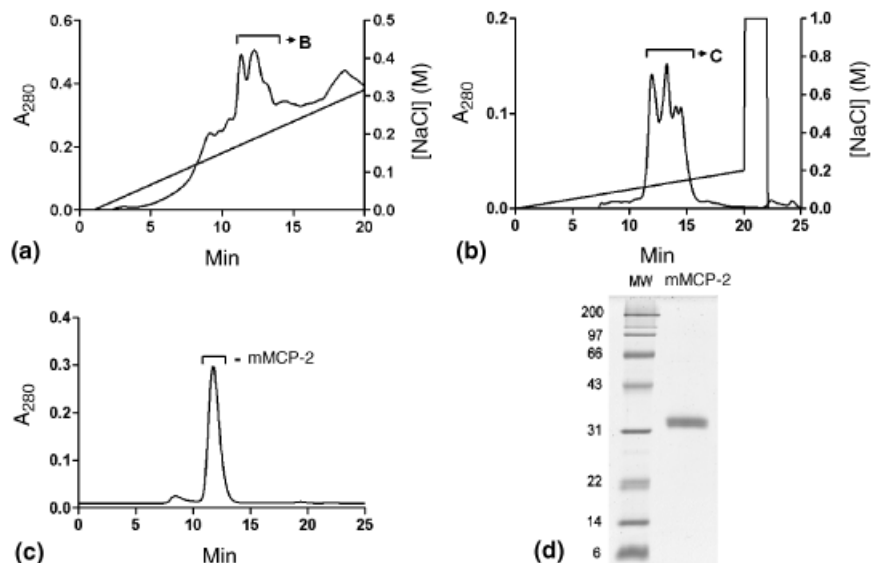


Fig. 1. Purification of mMCP-2. Homogenate from mMCP-1^{-/-} BMMC (see Materials and methods) was applied to a Mono S column in 50 mM MES, pH 6.1, 0.1% Brij 35. The column was eluted in a 0–0.5 M NaCl gradient, of which the 0–0.3 M section is shown. (a) The fractions indicated were combined and re-run on Mono S in 20 mM Tris-HCl, pH 7.5, 0.1% Brij 35 (b) in a 0–0.2 M NaCl gradient. The fractions indicated in (b) were combined, concentrated and finally purified by gel filtration (c) in PBS, 0.1% Brij 35. Purified mMCP-2 appeared as a diffuse doublet of approx 33–34 kDa by SDS-PAGE (d).

derived from mMCP-1^{-/-} mice [10, 11, 21, 22]. Following Mono S chromatography (Fig. 1a), SDS-PAGE analysis (not shown) of fractions revealed the presence of a major product with MW similar to that expected for mMCP-2, but with no detectable chymase activity. Further analysis of the putative mMCP-2 band by N-terminal amino acid sequencing (10 residues) showed that it was identical to that expected for mMCP-2 [19]. Re-chromatography of the mMCP-2-positive fractions at pH 7.5 showed a tendency to separate into distinct forms (Fig. 1b). These were combined and finally purified by gel filtration, eluting with an apparent MW of approximately 35 kDa (Fig. 1c), based on prior MW calibration of the column. The typical final yield of mMCP-2 was approximately 400 µg from 1.4×10^8 cells (= 2.8 pg/cell). The product appeared by SDS-PAGE (Fig. 1d) to be a broad doublet at 33–34 kDa.

mMCP-2 substrate specificity

As described by Serafin et al. [19], mMCP-2 reacted with [³H]-labelled DFP (Fig. 2a). A strong signal was observed at 33–34 kDa, consistent with the molecular weight of mMCP-2, with intensity similar to that of a trypsin positive control, indicating the presence of an active catalytic triad in mMCP-2. However, of the chromogenic substrates tested, mMCP-2 only cleaved succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester at a detectable level. Even in this case, the specific activity of mMCP-2 was extremely low compared to chymotryptic proteinases of varying activity (Table 1), including prostate-specific antigen (kallikrein 3) which is renowned for its poor reactivity with chromogenic substrates [26]. All traces of chymotryptic activity were eliminated by treatment with DFP (data not shown). Additionally, while the universal protease substrate, resorufin-labelled casein, was readily cleaved by mMCP-1, incubation with mMCP-2 gave no reaction (Fig. 2b).

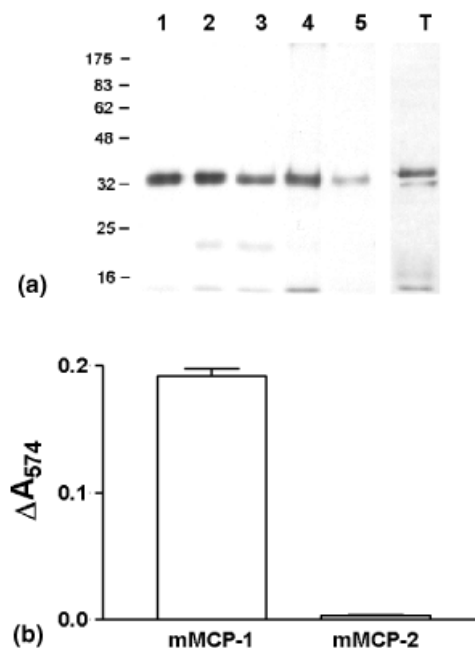


Fig. 2. Activity of mMCP-2. (a) [³H]-DFP binding. Autoradiograph of SDS-gel showing mMCP-2 (500 ng) incubated with [³H]DFP in the presence of (1) PBS (pH 7.4), (2) 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.2 M glycerol, (3) 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.2 M glycerol, 100 µg/mL heparin, (4) 20 mM Tris-HCl pH 8.0, 2 M NaCl, 0.2 M glycerol, (5) 20 mM MOPS pH 6.8, 0.15 M NaCl, 0.2 M glycerol. Positive control lane (T) contained equine trypsin (525 ng) in buffer (3). (b) Hydrolysis of universal protease substrate (resorufin-labelled casein; 100 µg/mL) in PBS with mMCP-1 and -2 (38 µg/mL) at 37 °C for 2 h. Label freed by proteolytic activity was assayed at 574 nm, in pH 8.8 buffer, following TCA precipitation of proteins. Values shown are corrected for control PBS incubation. mMCP-2 values were not statistically different from control ($n = 3$).

Comparative immunolocalization of mMCP-1 and -2

Previous studies [6] established that a proportion of gastric mast cells in normal BALB/c mice did not express mMCP-1,

Table 1. Specific activities of mMCP-2 and selected chymotryptic proteinases towards a chromogenic peptide substrate

Enzyme	Specific activity (U/mg)	Relative specific activity
mMCP-2	0.041	1
Human prostate-specific antigen	0.39	9
Human cathepsin G	9.4	230
Human chymase	197	4740
mMCP-1	423	10200

Samples were assayed with 0.5 mM succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester in 0.1 M DTNB, 1.5 M NaCl, 0.3 M Tris-HCl, pH 8.0. One unit (U) of activity is defined as the amount of enzyme required to hydrolyse one micromole of substrate in 1 min.

but after nematode infection almost 100% of gastric MMC were mMCP-1 positive (mMCP-1^{+ve}). The comparative expression of mMCP-2 by gastric MMC was therefore investigated. Rat anti-mMCP-2 antibodies detected mast cells in the stomach of uninfected BALB/c mice (Fig. 3a), whereas monoclonal rat anti-mMCP-1 detected far fewer cells (Fig. 3b) and control rat IgG was negative (Fig. 3c). Ear

sections contained abundant toluidine blue-positive mast cells (Fig. 3d), which are known in the BALB/c strain to contain high levels of transcripts for mMCP-4 and -5, and for the tryptases, mMCP-6 and -7, but not for mMCP-1 and -2 [27]. Mast cells were not detected in these sections by rat or sheep anti-mMCP-2 antibodies (Figs 3e and f), indicating that these reagents do not cross-react with mMCP-4, -5, -6 or -7. The distribution of mMCP-1^{+ve} mast cells in the jejunum of *N. brasiliensis*-infected mice (Fig. 3g, and see below) was as previously described, with positive cells predominantly located within the epithelium [4, 6]. The distribution of mMCP-2^{+ve} cells was very similar, using both rat and sheep anti-mMCP-2 antibodies (Figs 3h and i, respectively).

ELISA measurement of the differential systemic release of mMCP-1 and -2 in normal mice

The mMCP-2 ELISA was specific, sensitive (to 0.1 ng/mL), and unaffected by the presence of mouse serum (Fig. 4a). The %CV (intra-assay) was 6.9% ($n = 7$), and %CV (inter-assay) was 24.9% ($n = 7$). The latter was rather high, reflecting the high dilution factor (80 000-fold) required for the chosen positive-control jejunal homogenate. The specificity of the

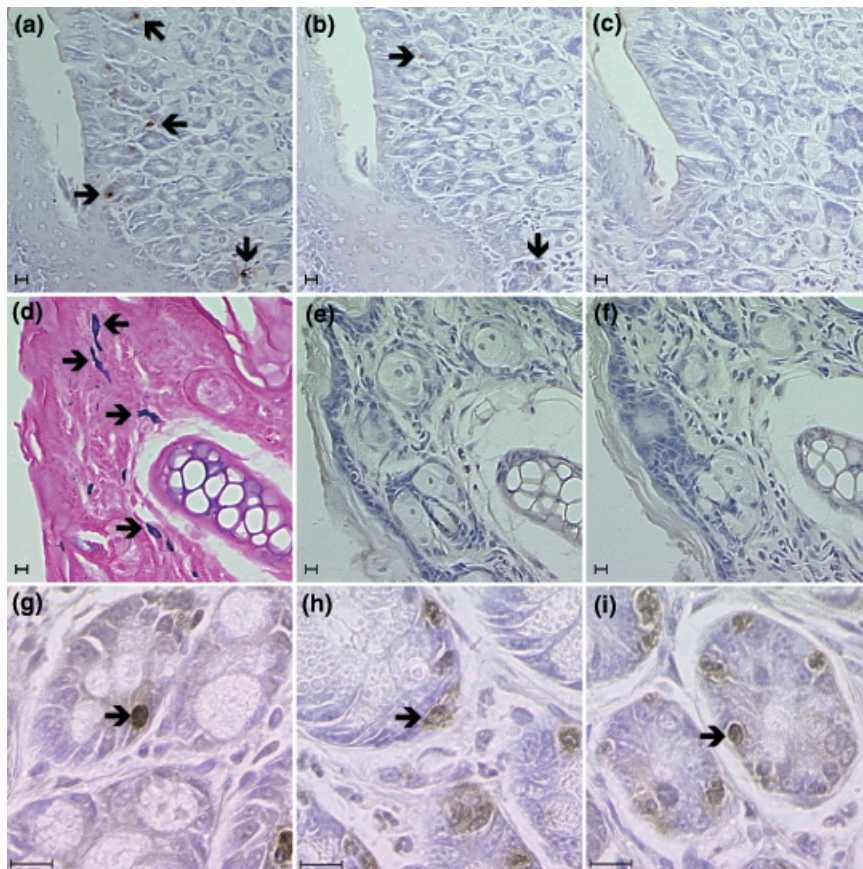


Fig. 3. Immunohistochemical detection of mouse mast cells. Tissues from uninfected BALB/c mice were fixed in 4% paraformaldehyde and processed as described in Materials and methods. Serial sections (4 μ m) of stomach (a–c) were probed with (a) rat anti-mMCP-2 Ig, (b) monoclonal rat anti-mMCP-1 and (c) control rat IgG. Positive staining is observed with the anti-mMCP-2 Ig, but with anti-mMCP-1, there are only occasional weakly stained cells (arrows). Control IgG sections were negative. Serial sections (4 μ m) of ear pinna were stained with (d) toluidine blue (pH 0.5)/eosin to reveal connective tissue mast cells, (e) rat anti-mMCP-2 Ig or (f) affinity-purified sheep anti-mMCP-2 Ig. Rat and sheep polyclonal anti-mMCP-2 Ig do not stain connective tissue mast cells. In the jejunum of *N. brasiliensis*-infected mice, there were abundant immunoreactive mast cells within the mucosal epithelium, when detected with monoclonal rat anti-mMCP-1 (g), polyclonal rat anti-mMCP-2 (h) and affinity-purified sheep anti-mMCP-2 Ig (i). Scale bars represent 10 μ m.

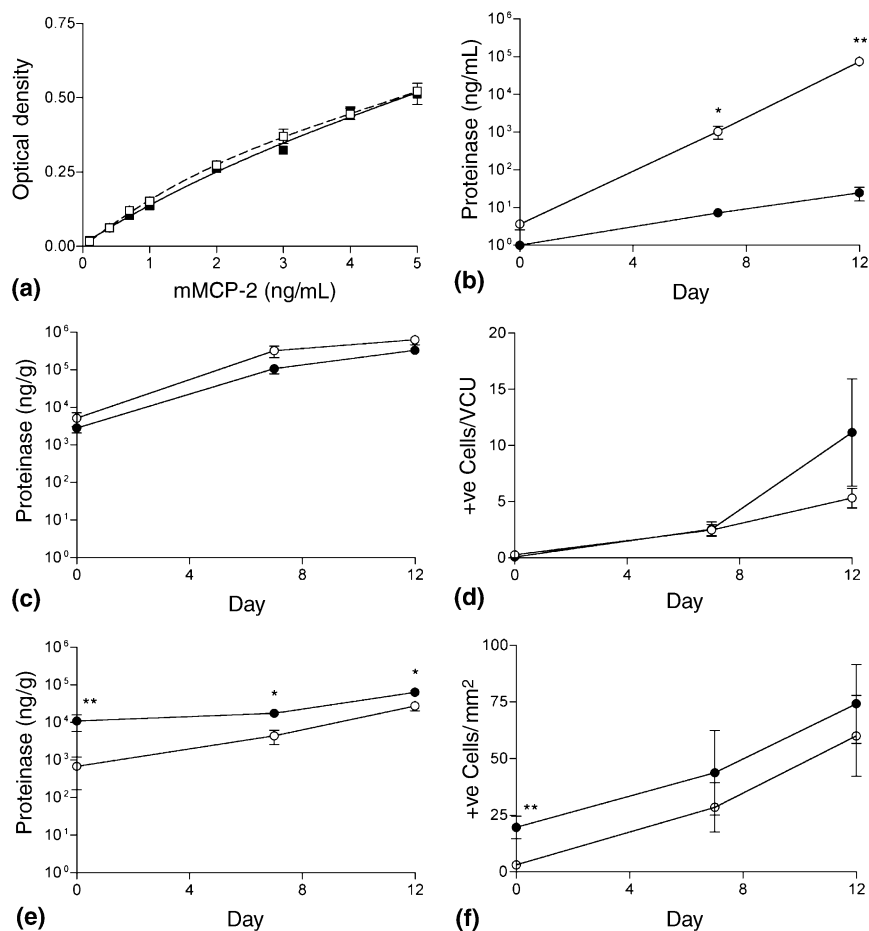


Fig. 4. mMCP-1 and -2 expression during *N. brasiliensis* infection. (a) Typical standard curves for the mMCP-2 ELISA where the mMCP-2 standard was incubated with (filled squares) and without (clear squares) normal mouse serum. The presence of serum did not affect the standard curve. BALB/c mice were infected with *N. brasiliensis* and tissues collected at days 7 and 12 of infection for analysis by ELISA and immunohistochemistry. ELISA data for mMCP-1 (clear circles) and mMCP-2 (filled circles) are presented as mean values $\pm$ SEM ($n = 5$) with units of ng/mL in the case of serum (b), or ng/g wet weight tissue in the case of the jejunum (c) and stomach (e). Immunoreactive cells in the jejunum (d) and stomach (f) are expressed as positive cells per villus-crypt unit (VCU) in the case of the jejunum, and positive cells per mm² in the case of the stomach.

ELISAs was tested by assaying purified mMCP-2 (1 μ g/mL) in the mMCP-1 ELISA and *vice versa*. In the mMCP-1 ELISA, 1 μ g/mL mMCP-2 was below the level of detection (0.5 ng/mL), indicating less than 0.05% cross-reactivity. In the mMCP-2 ELISA, the original mMCP-1 sample gave 5% cross-reactivity, due to contaminating mMCP-2. Further purification of the mMCP-1 sample by heparin affinity chromatography and selection of the leading edge fraction (not shown) reduced the level of cross-reaction to 0.1%. Therefore, both ELISAs were specific for their respective analytes.

Baseline values for the relative expression of mMCP-1 and -2 were obtained, and Table 2 shows the comparative results for mMCP-1 and -2 ELISAs performed on tissues from uninfected BALB/c mice. Traces of mMCP-1 were detectable in normal serum, but mMCP-2 levels were below the detection limit (<0.1 ng/mL). Neither mMCP-1 nor mMCP-2 could be detected in extracts of the ear. However, both were detected in the stomach, with basal levels of mMCP-2 being significantly higher than those of mMCP-1. The jejunum contained both mMCP-1 and -2 at comparable absolute concentrations (Table 2).

Table 2. Concentration of mMCP-1 and -2 in tissues from uninfected BALB/c mice

Tissue type	mMCP-1	mMCP-†
Serum (ng/mL)	2.6 $\pm$ 1.0*	ND
Ear (ng/g)	N.D.	N.D.
Stomach (ng/g)	6.8 $\pm$ 5.2 $\times 10^2$	1.1 $\pm$ 0.5 $\times 10^4$ ‡
Jejunum (ng/g)	5.2 $\pm$ 2.1 $\times 10^3$	2.8 $\pm$ 0.7 $\times 10^3$

*Mean $\pm$ SEM; $n = 5$.

†ND = not detected.

‡Significantly more mMCP-2 than -1 was detected in the extracts of uninfected mouse stomach ($P = 0.0079$; Mann-Whitney test).

Differential expression and systemic release of mMCP-1 and -2 during infection with *N. brasiliensis*

During infection with intestinal nematodes, MMC hyperplasia is associated with very substantial increases in the levels of mMCP-1 in both the GI tract [6, 14] and in the peripheral blood [6, 8]. We therefore compared the expression and systemic release of mMCP-1 and -2 in groups of BALB/c mice infected with *N. brasiliensis*. Fecal egg counts peaked at

day 7 ($13\,900 \pm 600$ eggs per gram of feces, mean $\pm$ SEM), and then returned to baseline by day 12 (data not shown). While there was minimal mMCP-1 and no mMCP-2 in serum at day 0, mMCP-2 was detectable in serum on days 7 and 12 of infection (6.3 ± 1.8 and 23 ± 10 ng/mL, respectively; Fig. 4b). mMCP-1 was approximately three orders of magnitude more abundant in serum (1.0 ± 0.9 and 75 ± 20 μ g/mL at days 7 and 12, respectively; Fig. 4b). As expected, mMCP-1 levels in the jejunum were high at days 7 and 12 of infection (Fig. 4c), and comparable concentrations were observed for mMCP-2. Numbers of mMCP-1⁺ve and mMCP-2⁺ve MMC increased accordingly (Fig. 4d). As observed in uninfected mice, significantly more mMCP-2 than -1 was detected in the stomach of mice at days 7 and 12 of infection, concomitant with a rise in the concentration of both analytes (Fig. 4e). This was reflected by a significantly higher count of mMCP-2⁺ve than mMCP-1⁺ve MMC in the uninfected stomach (Fig. 4f), but the difference was not significant at days 7 and 12 of infection. These data show that while mMCP-2 is abundantly expressed by jejunal MMC, it is released systemically at much lower levels than mMCP-1.

Discussion

The expression of mast cell proteinases is known to be crucially dependent on the local cytokine environment [28], such that subsets from intestinal mucosa and sub-mucosa of mice have quite different proteinase expression patterns [7]. We have previously shown that mMCP-1 and mMCP-2 mRNA transcripts are substantially up-regulated following treatment of mBMMC with TGF- β_1 and, crucially, that mMCP-1 is synthesized and secreted at comparable levels to those found *in vivo* [21, 22]. In the present study, we have taken advantage of the mMCP-1^{-/-} mouse [10, 11] to generate highly purified mMCP-2 from mBMMC grown in the presence of TGF- β_1 . In addition to facilitating the analysis of the substrate specificity of mMCP-2, this has enabled us to generate highly specific polyclonal antibodies with which to study the expression and release of this MMC proteinase *in vivo*.

We have verified that during nematode infection, there is a profound increase in the number of mMCP-1 and -2⁺ve cells in the jejunum, and shown that absolute levels of both proteinases, as detected by ELISA, increase accordingly. The situation in the gastric mucosa is different, where there are significantly more mMCP-2⁺ve than mMCP-1⁺ve MMC in uninfected mice. This distinction is also detectable by ELISA, and is maintained during *N. brasiliensis* infection, although in terms of cell counts, there was no significant difference in the number of mMCP-1⁺ve and -2⁺ve MMC during the course of infection. This would suggest that elevated expression of mMCP-2, relative to mMCP-1, occurs in the gastric mucosa even during infection. Whilst it seems likely that the expression of mMCP-2 is, like mMCP-1 [21, 22], regulated by the multifunctional cytokine TGF- β_1 , it is possible that additional regulatory signals operate within the gastric mucosa, which mediate the differential expression of mMCP-1 and -2 in this location. Alternatively, the expression and translation of mMCP-2 may be induced at lower concentrations of TGF- β_1 than mMCP-1.

The most marked difference in the distribution of mMCP-1 and -2 was in their relative abundance in the peripheral bloodstream. During infection, mMCP-1 levels rose dramatically, as is well documented [5]. However, mMCP-2 concentrations rose only modestly, to a level at least 1000-fold lower than mMCP-1, despite being expressed at almost identical levels to mMCP-1 in the jejunum and at higher levels in the gastric mucosa. There are several possible explanations for this discrepancy. *In vitro* MMC-homologues constitutively express and secrete large amounts of mMCP-1 in the presence of TGF- β_1 via the Golgi complex [29]. One explanation, therefore, may be that mMCP-2 is preferentially sequestered within the mast cell granules. Alternatively, mMCP-2 may be cleared from the peripheral bloodstream more rapidly than mMCP-1. These possibilities are currently under investigation.

While this study focuses attention on the expression and release of mMCP-1 and -2, their physiological targets and respective roles *in vivo* remain to be elucidated. The current study has failed to demonstrate any significant proteolytic activity for mMCP-2. This may be a consequence of the unique two amino acid deletion in the region of the S1 substrate-binding pocket of mMCP-2 [19], centred on residue 216 (standard chymotrypsinogen numbering), which may modify the geometry of the pocket and its accessibility to amino acid side chains. The presence of a bulky amino acid side chain at position 216 is generally associated with elastase-like activity, and the existence of valine in this position in mMCP-5 has been shown to confer elastase-like properties [20]. Site-directed mutagenesis of mMCP-5, replacing Val216 with a glycine residue more typical of chymotrypsin-like enzymes, generated chymase-like activity [20]. The two amino acid deletion in mMCP-2 results in a tyrosine residue occupying the position equivalent to residue 216. This would block access to the bulky aromatic side chains of chymase substrates, but does not apparently confer elastase activity, since mMCP-2 was also inactive against elastase substrates. Interestingly, a mutation at residue 216 in human α_1 -tryptase also results in an almost complete loss of activity, but with retention of DFP-binding ability, due to the induction of a kinked conformation in the substrate-binding pocket [30]. Potential roles for functionally crippled mast cell proteinases remain speculative, but they may include anti-microbial activity, as is known for the inactive neutrophil elastase homologue, azurocidin [31].

At the outset of this study, it was known that mMCP-1 and -2 are similar in terms of sequence [19] and that they are both expressed in intestinal MMC [6, 32]. However, we conclude that they are not functionally redundant, since we have shown here that they differ markedly in activity, in their expression in the gastric mucosa and in their secretion into and/or half-life within the peripheral bloodstream. Identifying the different proteolytic targets of mMCP-1 and -2 and the mechanisms underlying their differential abundance in serum, therefore, remain as major goals in understanding the role of mucosal mast cells in the cell-mediated immune response of the gastrointestinal tract.

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References

- 1 Miller HRP. Mucosal mast cells and the allergic response against nematode parasites. *Vet Immunol Immunopathol* 1996; 54:331–6.
- 2 Dillon SB, MacDonald TT. Functional properties of lymphocytes isolated from murine small intestinal epithelium. *Immunology* 1984; 52:501–9.
- 3 Arizono N, Yamada M, Tegoshi T, Okada M, Uchikawa R, Matsuda S. Mucosal mast cell proliferation following normal and heterotopic infections of the nematode *Nippostrongylus brasiliensis* in rats. *APMIS* 1994; 102:589–96.
- 4 Miller HRP, Huntley JF, Newlands GF, Mackellar A, Lammas DA, Wakelin D. Granule proteinases define mast cell heterogeneity in the serosa and the gastrointestinal mucosa of the mouse. *Immunology* 1988; 65:559–66.
- 5 Wastling JM, Scudamore CL, Thornton EM, Newlands GF, Miller HRP. Constitutive expression of mouse mast cell protease-1 in normal BALB/c mice and its up-regulation during intestinal nematode infection. *Immunology* 1997; 90:308–13.
- 6 Scudamore CL, McMillan L, Thornton EM, Wright SH, Newlands GF, Miller HRP. Mast cell heterogeneity in the gastrointestinal tract: variable expression of mouse mast cell protease-1 (mMCP-1) in intraepithelial mucosal mast cells in nematode-infected and normal BALB/c mice. *Am J Pathol* 1997; 150:1661–72.
- 7 Friend DS, Ghildyal N, Gurish MF et al. Reversible expression of tryptases and chymases in the jejunal mast cells of mice infected with *Trichinella spiralis*. *J Immunol* 1998; 160:5537–45.
- 8 Tuohy M, Lammas DA, Wakelin D, Huntley JF, Newlands GF, Miller HRP. Functional correlations between mucosal mast cell activity and immunity to *Trichinella spiralis* in high and low responder mice. *Parasite Immunol* 1990; 12:675–85.
- 9 Irvine J, Newlands GF, Huntley JF, Miller HRP. Interaction of murine intestinal mast cell proteinase with inhibitors (serpins) in blood; analysis by SDS-PAGE and Western blotting. *Immunology* 1990; 69:139–44.
- 10 Wastling JM, Knight P, Ure J et al. Histochemical and ultrastructural modification of mucosal mast cell granules in parasitized mice lacking the beta-chymase, mouse mast cell protease-1. *Am J Pathol* 1998; 153:491–504.
- 11 Knight PA, Wright SH, Lawrence CE, Paterson YY, Miller HRP. Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *J Exp Med* 2000; 192:1849–56.
- 12 Gurish MF, Nadeau JH, Johnson KR et al. A closely linked complex of mouse mast cell-specific chymase genes on chromosome 14. *J Biol Chem* 1993; 268:11372–9.
- 13 Hunt JE, Friend DS, Gurish MF et al. Mouse mast cell protease 9, a novel member of the chromosome 14 family of serine proteases that is selectively expressed in uterine mast cells. *J Biol Chem* 1997; 272:29158–66.
- 14 Newlands GF, Gibson S, Knox DP, Grecis R, Wakelin D, Miller HRP. Characterization and mast cell origin of a chymotrypsin-like proteinase isolated from intestines of mice infected with *Trichinella spiralis*. *Immunology* 1987; 62:629–34.
- 15 Newlands GF, Knox DP, Pirie_Shepherd SR, Miller HRP. Biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4). *Biochem J* 1993; 294:127–35.
- 16 Serafin WE, Sullivan TP, Conder GA et al. Cloning of the cDNA and gene for mouse mast cell protease 4. Demonstration of its late transcription in mast cell subclasses and analysis of its homology to subclass-specific neutral proteases of the mouse and rat. *J Biol Chem* 1991; 266:1934–41.
- 17 Caughey GH, Raymond WW, Wolters PJ. Angiotensin II generation by mast cell alpha- and beta-chymases. *Biochim Biophys Acta* 2000; 1480:245–57.
- 18 Chandrasekharan UM, Sanker S, Glynias MJ, Karnik SS, Husain A. Angiotensin II-forming activity in a reconstructed ancestral chymase. *Science* 1996; 271:502–5.
- 19 Serafin WE, Reynolds DS, Rogelj S et al. Identification and molecular cloning of a novel mouse mucosal mast cell serine protease. *J Biol Chem* 1990; 265:423–29.
- 20 Kunori Y, Koizumi M, Masegi T et al. Rodent alpha-chymases are elastase-like proteases. *Eur J Biochem* 2002; 269:5921–30.
- 21 Miller HRP, Wright SH, Knight PA, Thornton EM. A novel function for transforming growth factor-beta 1: upregulation of the expression and the IgE-independent extracellular release of a mucosal mast cell granule-specific beta-chymase, mouse mast cell protease-1. *Blood* 1999; 93:3473–86.
- 22 Wright SH, Brown J, Knight PA et al. Transforming growth factor-beta1 mediates coexpression of the integrin subunit alphaE and the chymase mouse mast cell protease-1 during the early differentiation of bone marrow-derived mucosal mast cell homologues. *Clin Exp Allergy* 2002; 32:315–24.
- 23 Pemberton AD, McEuen AR, Scudamore CL. Characterisation of tryptase and a granzyme H-like chymase isolated from equine mastocytoma tissue. *Vet Immunol Immunopathol* 2001; 83:253–67.
- 24 McEuen AR, He S, Brander ML, Walls AF. Guinea pig lung tryptase. Localisation to mast cells and characterisation of the partially purified enzyme. *Biochem Pharmacol* 1996; 52:331–40.
- 25 McEuen AR, Gaça MD, Buckley MG, He S, Gore MG, Walls AF. Two distinct forms of human mast cell chymase – differences in affinity for heparin and in distribution in skin, heart, and other tissues. *Eur J Biochem* 1998; 256:461–70.
- 26 Huang X, Knoell CT, Frey G et al. Modulation of recombinant human prostate-specific antigen: activation by Hofmeister salts and inhibition by azapeptides. Appendix: thermodynamic interpretation of the activation by concentrated salts. *Biochemistry* 2001; 40:11734–41.
- 27 Stevens RL, Friend DS, McNeil HP, Schiller V, Ghildyal N, Austen KF. Strain-specific and tissue-specific expression of mouse mast cell secretory granule proteases. *Proc Natl Acad Sci USA* 1994; 91:128–32.
- 28 Miller HRP, Pemberton AD. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 2002; 105:375–90.
- 29 Brown JK, Knight PA, Wright SH, Thornton EM, Miller HRP. Constitutive secretion of the granule chymase mouse mast cell protease-1 and the chemokine, CCL2, by mucosal mast cell homologues. *Clin Exp Allergy* 2003; 33:132–46.
- 30 Marquardt U, Zettl F, Huber R, Bode W, Sommerhoff C. The crystal structure of human alpha1-tryptase reveals a blocked substrate-binding region. *J Mol Biol* 2002; 321:491–502.
- 31 Wilde CG, Snable JL, Griffith JE, Scott RW. Characterization of two azurphil granule proteases with active-site homology to neutrophil elastase. *J Biol Chem* 1990; 265:2038–41.
- 32 Friend DS, Ghildyal N, Austen KF, Gurish MF, Matsumoto R, Stevens RL. Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J Cell Biol* 1996; 135:279–90.